Human Eccrine Sweat Gland Cells Can Reconstitute a Stratified Epidermis

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Eccrine sweat glands are generally considered to be a possible epidermal stem cell source. Here we compared the multilayered epithelia formed by epidermal keratinocytes and those formed by eccrine sweat gland cells. We demonstrated both *in vitro* and *in vivo* the capability of human eccrine sweat gland cells to form a stratified interfollicular epidermis substitute on collagen hydrogels. This is substantiated by the following findings: (1) a stratified epidermis consisting of 10–12 cell layers is formed by sweat gland cells; (2) a distinct stratum corneum develops and is maintained after transplantation onto immuno-incompetent rats; (3) proteins such as filaggrin, loricrin, involucrin, envoplakin, periplakin, and transglutaminases I and III match with the pattern of the normal human skin; (4) junctional complexes and hemidesmosomes are readily and regularly established; (5) cell proliferation in the basal layer reaches homeostatic levels; (6) the sweat gland-derived epidermis is anchored by hemidesmosomes within a well-developed basal lamina; and (7) palmo-plantar or mucosal markers are not expressed in the sweat gland-derived epidermis. These data suggest that human eccrine sweat glands are an additional source of keratinocytes that can generate a stratified epidermis. Our findings raise the question of the extent to which the human skin is repaired and/or permanently renewed by eccrine sweat gland cells.

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INTRODUCTION

Keratinocyte stem cells are supposed to assure the homeostatic renewal of the epidermis and to be responsible for re-epithelialization of the epidermis after skin injuries. Keratinocyte stem cells reside not only in the epidermis but also in appendageal structures protruding into the dermis. Outer root sheath cells of hair follicles were shown to be able to regenerate a fully differentiated epidermis *in vitro* and were used for the production of epidermal equivalents for the treatment of skin damage or disease (Lenoir *et al.*, 1988; Limat *et al.*, 1996, 2003).

The bulge region of both mouse and human hair follicles has been shown to contain a multipotent stem cell population (reviewed in Alonso and Fuchs, 2003; Cotsarelis, 2006). In the mouse, these stem cells do not appear to contribute to epidermal regeneration under homeostatic conditions (Ito *et al.*, 2005; Levy *et al.*, 2005). It remains to be investigated whether this is also true for the human skin. Interestingly, human glabrous skin, which is completely free of hair follicles but contains a high number of sweat glands (Frinkel and Woodley, 2001), also exhibits regenerative potential. This fact prompted us to ask whether sweat gland-derived epithelial cells can differentiate into keratinocytes and form an epidermis.

Human eccrine sweat ducts develop at around gestational week 15–20 by budding from the basal cell layer. The resulting anlagen protrude into the dermis, forming the globular secretory domain (Moll and Moll, 1992; Ersch and Stallmach, 1999). As very little is known about the differentiation of sweat glands, any effort to regenerate them *in vitro* remains extremely ambitious (Shikiji *et al.*, 2003). There are some indications that sweat gland cells may have a certain capacity to form an epithelium in culture and *in vivo* (Lobitz *et al.*, 1954; Jones *et al.*, 1988; Miller *et al.*, 1998). Yet, these few studies did not address the issue of the type and quality of the epithelium produced.

The presence of a sufficient number of viable epidermal stem cells in cultured epidermal autografts is of major clinical importance (O'Connor, 1981; Auger *et al.*, 2004; De Luca *et al.*, 2006; MacNeil, 2007). We reasoned that human eccrine sweat glands may be an additional source of epidermal stem cells, which may contribute to the *in vitro* maintenance and long-term survival and function of engineered epidermal grafts.

To address this issue, we raised organotypic cultures consisting of a dermal component and a stratified epidermal substitute (ES) formed by epidermal keratinocytes and sweat gland cells, respectively. These substitutes, designated KdES (keratinocyte-derived ES) and SdES (sweat gland-derived ES), respectively, were transplanted onto the backs

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Abbreviations: ES, epidermal substitute; K, (cyto)keratin; KdES, keratinocyte-derived epidermal substitute; SdES, sweat gland-derived epidermal substitute

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of immuno-incompetent rats and the expression of protein markers indicative of the grade of differentiation and homeostasis was determined. Although with a delay in comparison with epidermal keratinocytes, sweat gland-derived cells developed ''*de novo*'' into a functional stratified, interfollicular epidermis.

RESULTS

Sweat gland-derived epithelial cells acquire the properties of epidermal keratinocytes in engineered dermo-epidermal substitutes

Human eccrine sweat glands were isolated from the scalp, abdomen, and the retro-auricular area of young patients ranging in age from 1 to 18 years. As depicted in Figure 1a, preparations of glandular structures consisted of the secretory domain (white arrowheads), the intraglandular duct, and stretches of the intradermal duct of varying length (black arrows). Isolated intradermal ducts always lacked the intraepidermal acrosyringium and a significant stretch of the upper duct (Figure 1a). Consequently, epidermal cells were avoided in sweat gland-derived cell preparations. Keratinocytes were isolated mainly from foreskins. Both cell types were expanded during passage 1. In Figure 1b, the outgrowth of the above-mentioned three domains of an eccrine sweat gland is depicted. Myoepithelial cells were associated with secretory sweat gland cells and were co-isolated. They were detected by smooth muscle actin expression and shown to disappear from the culture within 5–7 days (data not shown).

In the subsequent experiments, sweat gland-derived (three-dimensional) cell cultures were compared with the corresponding cultures of keratinocytes. The cells were initially seeded within a ring of 0.7 cm^2, and were grown on collagen type I hydrogels that had been previously populated with 1×10^5 primary human dermal fibroblasts. Keratinocytes finally covered the entire gel, thereby showing a surface expansion of about 4 times (Figure 1c). Sweat gland cells expanded somewhat slower, increasing the surface of the initial cell layer by approximately 2.5 times (Figure 1d).

After the *in vitro* culture phase, the composite substitutes consisting of a dermal and a multilayered epidermal equivalent were transplanted onto the backs of immuno-incompetent Nu/Nu rats. All the subsequent comparative analyses were performed on histological sections of dermo-epidermal grafts after their transplantation onto immuno-incompetent rats. To prevent the ingrowth of rat-derived skin cells, a transplantation chamber was used as previously described (Pontiggia *et al.*, 2009; Figure 1e and f). Histological analyses 21 days after transplantation revealed that both epidermal keratinocytes and sweat gland cells formed a stratified epidermis, which consisted of a basal layer, 10–12 suprabasal layers, and a well-differentiated anuclear stratum corneum (Figure 1g and h).

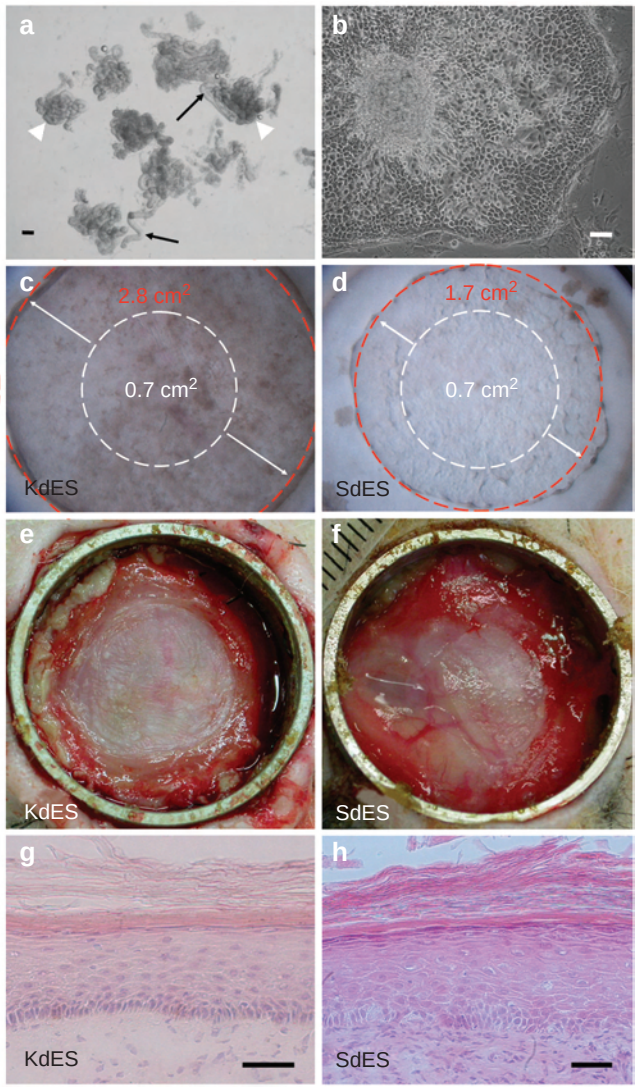

Figure 1. Macroscopic and histological appearance of sweat gland-derived epidermal substitutes (SdESs) and keratinocyte-derived epidermal substitutes (KdESs) *in vitro* and after transplantation onto immuno-incompetent rats. (**a**) Isolation of human sweat glands after dispase/collagenase digestion. Sweat gland fragments containing the glandular domain (white arrowheads) and parts of the intradermal ducts (black arrows). (**b**) A typical epithelial cell colony growing out from a human sweat gland fragment. (**c**, **d**) Keratinocytes and sweat gland cells grown at the air–liquid phase on collagen type I hydrogels on permeable inserts. Keratinocytes were initially seeded within a ring area of 0.7 cm^2. At 3 weeks after plating, this area was increased by about 4 times. Sweat gland cells increased this area by approximately 2.5 times. (**e**, **f**) KdES (e) and SdES (f) 3 weeks after transplantation onto full-thickness skin wounds on the back of athymic rats. (**g**, **h**) Hematoxylin/eosin staining of KdES (**g**) and SdES (**h**) 3 weeks after transplantation reveals a stratified multilayer consisting of a basal layer, 10–12 suprabasal layers, and a stratum corneum in both types of substitutes. (**a**) Bar = 500 μm; (**b**) bar = 200 μm; (**g**, **h**) bar = 100 μm. The rings in (**e**) and (**f**) are 2.7 cm in diameter.

Characteristics of a stratified epidermis formed by sweat gland cells

Owing to the use of the microdissection technique, it is unlikely that our sweat gland cell preparations were contaminated by epidermal keratinocytes. Nevertheless, we performed control experiments to discriminate between keratinocytes and sweat gland cells in the initial cell preparations (Figure 2c and d) and in SdES and KdES (Figure 3a–f). As shown in Table 1, only a very limited selection of antibodies, such as antibodies to K2e and K8,

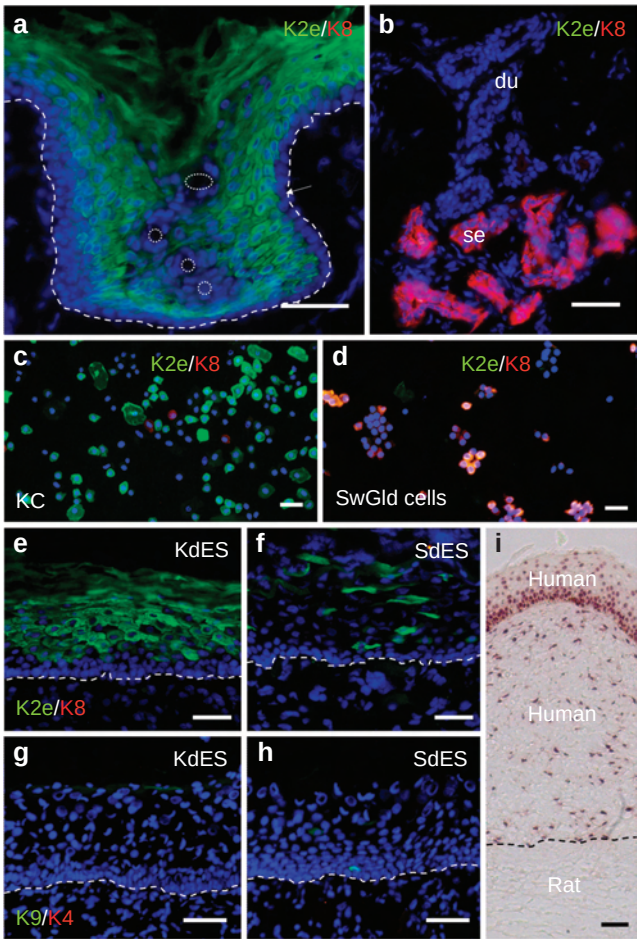

Figure 2. Tissue-specific markers in keratinocyte-derived epidermal substitutes (KdESs) and sweat gland-derived epidermal substitutes (SdESs). Double immuno-fluorescence using the antibody combinations K2e/K8 (**a–f**) and K9/K4 (**g**, **h**). (**a**) K2e is expressed in suprabasal layers (white arrowhead), as shown in the area of the glandular acrosyringium. Note that the sweat gland duct (dotted circles), meandering through the acrosyringium, does not express K2e. K8 is not expressed in normal human epidermis. (**b**) In normal human skin, the duct (du) of an eccrine sweat gland is negative for K8 (and K2e), whereas the secretory domain (se) expresses K8. (**c**) Cytospin of a freshly isolated human sweat gland cell suspension. About 50% of the cells are positive for K8. All sweat gland cells are negative for K2e. (**d**) In contrast, freshly isolated human keratinocytes are positive for K2e and negative for K8. (**e**) Immuno-fluorescence staining for the expression of K2e and K8 of KdES 3 weeks after transplantation. (**f**) Only a few single cells are staining for K2e/K8 in SdES. (**g**, **h**) Almost no expression of the palmo-plantar marker K9 and the mucosal marker K4 in KdES (**g**) and SdES (**h**) after transplantation. (**i**) *In-situ* hybridization of SdES using a DNA probe that hybridizes to human-specific Alu sequences. Positive (violet) staining proves the human origin of the dermo-epidermal graft. The boundary between human tissue and the underlying unstained rat tissue is obvious. White dotted line: dermo-epidermal border. Bar = 50 μm.

could be used for these analyses. K2e is expressed in all suprabasal keratinocytes of the human epidermis (Smith *et al.*, 1999), whereas it cannot be recognized in the sweat gland, not even in the intraepidermal acrosyringium (Figure 2a and b). As shown in Figure 2b, K8 expression is confined to the secretory domain of the sweat gland.

Cytospins of newly prepared primary sweat gland cells and keratinocytes were analyzed for the expression of K2e and K8. Importantly, none of the sweat gland cell preparations ($n = 3$) contained K2e-expressing cells, whereas about 50% were positive for K8 (Figure 2d). These findings indicate that the sweat gland cell preparations were not contaminated by (suprabasal) epidermal keratinocytes. In contrast, the majority of epidermal keratinocytes expressed K2e, whereas these cells did not stain for K8 (Figure 2c). Although epidermal keratinocytes completely downregulated K2e expression on cell culture plastic (data not shown), a significant number of them re-expressed K2e when integrated into differentiated ESs, whereas K8 was not expressed by epidermal keratinocytes (Figure 2e).

One significant difference between human eccrine sweat gland cells and epidermal keratinocytes was that only a relatively low number of sweat gland cells expressed K2e within 5–7 weeks in differentiating epidermal equivalents (Figure 2f). Notably, the number of K2e-expressing cells in SdES did not significantly increase 7–10 weeks after their transplantation (data not shown). Thus, the K2e-expressing cells in SdES may represent sweat gland-derived cells that underwent additional epidermal differentiation steps. It remains to be investigated, however, whether the number of K2e-positive sweat gland cells would still increase during an extended *in vivo* differentiation period of $\geqslant 3$ months. Notably, K8-positive cells were completely absent in SdES, indicating that this (secretory domain-specific) sweat gland marker was no longer expressed in the engineered epidermis (Figure 2f).

In the next experimental series we set out to explore whether the SdES exhibited mucosal or palmo-plantar characteristics. This was done by screening the two types of ESs for the expression of K4, a mucosal marker, and K9, a marker of palmo-plantar epidermis. In none of these experiments ($n = 4$) did we find expression of K4, indicating that there was no mucosal differentiation. Furthermore, K9-positive cells were detected neither in KdES nor in SdES ($n = 4$), showing that palmo-plantar properties were absent (Figure 2g and h).

To monitor the contribution of rat keratinocytes to the formation of the ES, we intended to confirm the human origin of the substitutes. Paraffin sections of engineered transplants were subjected to *in situ* hybridization using DNA probes specifically hybridizing to primate-specific Alu repeats. Figure 2i shows the well-defined transition zone (black dotted line) between the graft of human origin and the underlying, unstained rat tissue. Immunostaining for the human-specific nuclear antigen and for the human fibroblast specific CD90/Thy-1 antigen also confirmed the human origin of the analyzed tissue sections (data not shown).

As there was no reliable antibody allowing to discriminate between basal epidermal keratinocytes and sweat gland cells (see also Table 1), we used an indirect assay to determine whether cells of the epidermal stratum basale were ''contaminating'' the sweat gland cell fraction. This was achieved by screening the two different types of ESs (KdES and SdES) for the presence of epidermal melanocytes. Melanocytes are

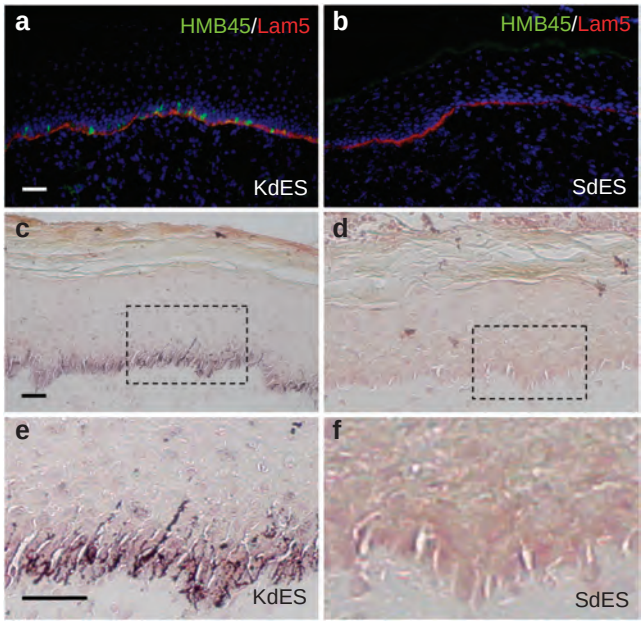

Figure 3. Preparations of human eccrine sweat gland epithelial cells are not contaminated by basal epidermal cells. (**a**) Histological section of a typical dermo-epidermal substitute (keratinocyte-derived epidermal substitute (KdES)) transplanted onto immuno-incompetent nu/nu rats 3 weeks after transplantation. Melanocytes (HMB45, green cells) located in the stratum basale are obvious. The basement membrane is stained by a laminin 332 antibody. (**b**) No melanocytes are detectable in sections of sweat gland-derived epidermal substitute (SdES), indicating that no basal epidermal cells, and hence no basal epidermal keratinocytes, were present in the initial sweat gland cell preparation. (**c**) Masson–Fontana staining reveals the melanin content both in basal melanocytes and in epidermal keratinocytes. (**d**) No melanosomes are detectable in SdES. (**e**) Magnified detail of (**c**). (**f**) Magnified detail of **d**. (**a**–**f**) Bar = 50 μm.

located in the epidermal stratum basale, and are always present in epidermal keratinocyte preparations, whereas they are absent in eccrine sweat glands. We were able to show that melanocytes were present in all KdESs investigated (Figure 3a), whereas no melanocytes could be detected in SdESs (Figure 3b). Accordingly, we showed that the transfer of melanin from melanocytes to keratinocytes takes place in KdES (Figure 3c and e), whereas this was not possible in SdES (Figure 3d and f).

Homeostasis of transplanted ESs

To determine the proliferative capacity of the epithelial cells in the ESs, frozen sections were stained for Ki-67, a nuclear cell proliferation-associated antigen, which is expressed in all active stages of the cell cycle (Gerdes *et al.*, 1991). In unwounded, homeostatic human skin, Ki-67 was expressed in single keratinocytes in the basal and first suprabasal layers in the interfollicular epidermis (see Supplementary Figure S1a online). We observed Ki-67-positive cells in the basal layer of both types of ESs (Figure 4a and b) 3 weeks after transplantation, indicating that the stratification process was ongoing and that, as in normal skin, only a small subpopulation of keratinocytes maintained epidermal renewal. An identical pattern of proliferative cells was observed in 7-week-old grafts (Figure 4c).

Normal, homeostatic interfollicular epidermis lacks the expression of K16 and K17 (Moll *et al.*, 1983; Troyanovsky *et al.*, 1989; see also Supplementary Figure S1a online). These keratins are induced in suprabasal, post-mitotic keratinocytes when tissue homeostasis is perturbed in wound-repair situations, such as the re-epithelialization after transplantation of dermo-epidermal substitutes or in cultures of dissociated keratinocytes grown on cell culture plastic (Leigh *et al.*, 1995; Paladini *et al.*, 1996; Coulombe, 1997). In both KdESs and SdESs, the expression of K16 was clearly detected in suprabasal cell layers soon after transplantation (Figure 4a and b), indicating a persistent wound-healing activity. At about 7–10 weeks after transplantation K16 was somewhat downregulated in both types of substitutes (Figure 4c; data not shown).

Stratification of engineered epidermal equivalents

Involucrin, envoplakin, and periplakin are markers of the cornification process and are associated with the formation of desmosomes and intermediate filaments (DiColandrea *et al.*, 2000). In normal human skin involucrin and loricrin are expressed in the upper granular and cornified layers (Supplementary Figure S1c online).

We found that the ''early'' pattern of involucrin expression differed from that of homeostatic skin in both types of substitutes. In fact, a strong expression was visible in the spinous layer 3 weeks after transplantation (Figure 4d and e). At 7 weeks after transplantation, as stratification and tissue homeostasis had further progressed, the lower suprabasal layers had downregulated involucrin expression (Figure 4f). The expression pattern of loricrin was similar to that of normal skin, both in the KdESs and in the SdESs, 3 weeks (Figure 4d and e) and 7 weeks (Figure 4f) after transplantation. Expression of envoplakin, filaggrin, and the transglutaminases I and III essentially confirmed the findings obtained with loricrin and involucrin (data not shown). Once again, the differences between KdESs and SdESs were moderate.

The distribution of K1/10, as late differentiation markers (Patel *et al.*, 2006), is indicative of the degree of tissue homeostasis in normal human skin. In fact, during the stratification process K10 appeared sooner in all suprabasal layers, whereas K1 expression was delayed and started in the more upper layers (Stark *et al.*, 1999). KdES (Figure 4g) eventually showed a staining pattern comparable to that of normal skin (see also Supplementary Figure S1d online). In general, the stratification process of the SdES was delayed in comparison with the KdES, with K1 being expressed after 3 weeks only in the upper stratum spinosum and granulosum (Figure 4h). In SdES the expression pattern of normal human skin was reached at about 7 weeks after transplantation (Figure 4i).

During stratification the epidermis establishes an intricate network of different intercellular junctional complexes to establish its functions as a barrier against external agents, as a water-balance regulator, and as a nutrient circulator (Morita

Table 1. The expression patterns of epithelial markers

		Human epidermis		Human sweat gland		
Marker	**Antibody**	**Suprabasal KC**	**Basal KC**	**Acro-syringium**	**Duct**	**Secretory domain**
CEA	Polyclonal	–	–	–	+	+
K1	LHK1	+	–	+	–	–
K5	Polyclonal	(+)	+	–	+	+
K8	B391	–	–	–	–	+
K10	DE-K10	+	–	+	+	–
K14	LL002	(+)	+	–	+	+
K16	LL025	+[1]	+[1]	n.d.	+	(+)
K19	RCK108	–	+[2]	+	+	+
Involucrin	SY5	+	–	+	+	–
K1b (K77)	Polyclonal	+	–	–	+	–
K2e	Ks 2.342.7.4	+	–	–	–	–
K15	LHK15, SPM190	–	+	–	–	+
K17	Ks 17.E3	–	–	–	+	+
K20	Ks 20.8	–	–	–	–	–
Desmoglein 1	27B2	+	–	n.d.	–	–
Desmoglein 3	5G11	+	+	n.d.	+	–
Connexin 26	CX-1E8	–	–	–	+	(+)
Integrin α6	4F10	–	+	–	+	+
Aquaporin 5	Polyclonal	–	+	n.d.	–	+
Follistatin	85918	–	+	–	–	+
CD200	MRC OX104	–	–	–	+	–
Na,K-ATPase α1	Polyclonal	+	+	n.d.	+	+
Transglut. 1	Polyclonal	+	–	n.d.	–	–
Transglut. 3	Polyclonal	+	–	n.d.	–	–
Transglut. 5	Polyclonal	+	+	n.d.	+	+

Abbreviations: KC, keratinocytes; n.d., not determined; +, expressed; –, not expressed; (+), minimal expression.
[1]Wound-healing marker.
[2]Newborn.

and Miyachi, 2003; Perez-Moreno *et al.*, 2003; Dusek *et al.*, 2007). We analyzed the presence of tight junctions, as, together with the lamellar bodies of the cornified envelope, these establish the epidermal diffusion barrier (Malminen *et al.*, 2003; Schluter *et al.*, 2004). One of the major constituents of tight junctions, occludin, is expressed in the upper granular layer of the human skin (Supplementary Figure S1e). Its typical expression pattern was obtained in both types of ES, 3 weeks after transplantation (Figure 4k and l). However, in the SdES we observed stronger and more homogeneous occludin expression only at 7 weeks after transplantation (Figure 4m).

E-cadherin is expressed as a component of adherens junctions in the living keratinocyte layers of the human epidermis (Moles and Watt, 1997; see also Supplementary Figure S1e online). Both types of ESs showed the expression pattern of a normal epidermis (Figure 4k, l, and m).

Desmosomes and the dermo-epidermal junction in engineered SdES

The desmosomal proteins desmoglein (Dsg) 1 and 3 are expressed in a graded manner in the human epidermis. Dsg 3 is expressed in the stratum basale, whereas Dsg 1 can be detected in the upper layers (Supplementary Figure S1f online). Dsg 1 and 3 and their partners desmocollin 1 and 3, respectively, are involved in the regulation of epidermal differentiation (Elias *et al.*, 2001; Dusek *et al.*, 2007). Dsg 3 is expressed exclusively in the ductal portion of the sweat gland (data not shown). During the first 3 weeks after transplantation both types of substitutes showed a somewhat unbalanced (mixed) expression pattern of Dsg 1 and Dsg 3. The suprabasal layers co-expressed both proteins in comparable quantities (Figure 5a and b). For the establishment of the physiological protein gradients, longer time periods (7 weeks and more) were necessary (Figure 5c).

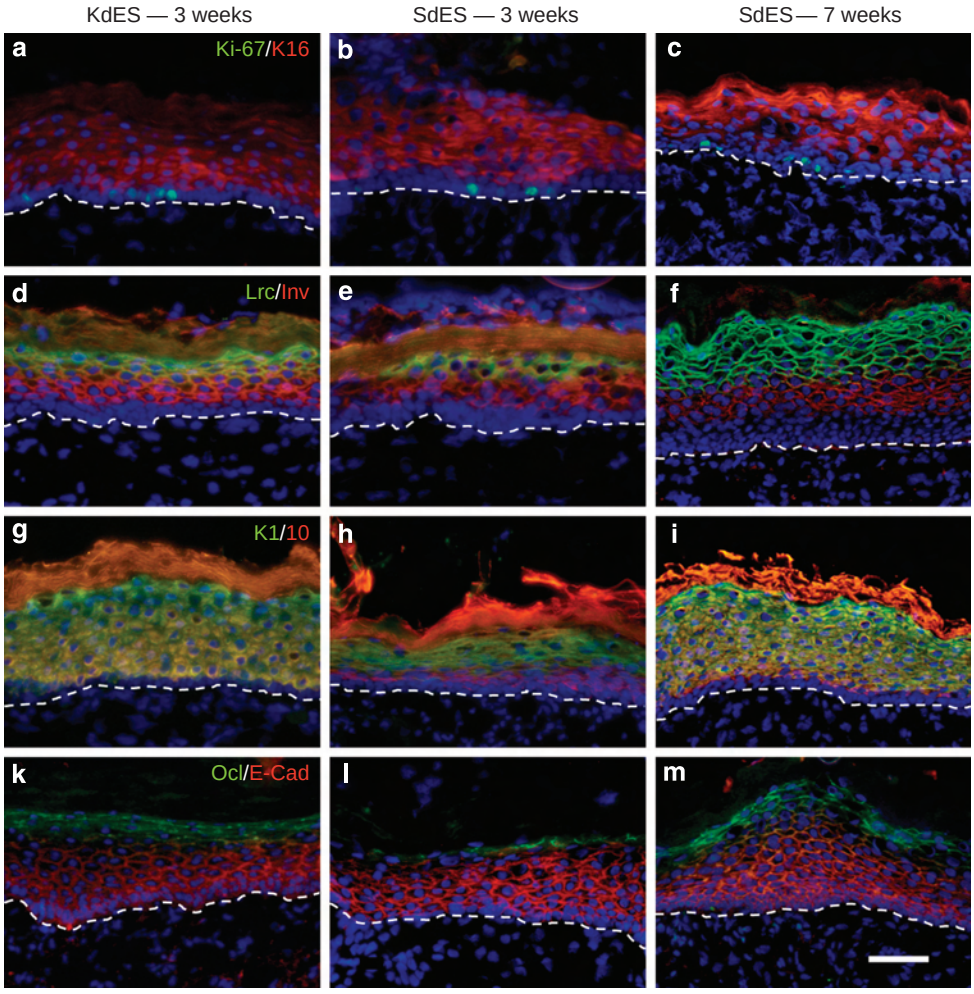

Figure 4. Epidermal stratification and homeostasis in keratinocyte-derived epidermal substitutes (KdESs) and sweat gland-derived epidermal substitutes (SdESs) after transplantation onto immuno-incompetent rats. Double immuno-fluorescence using antibodies to Ki-67/cytokeratin 16 (**a–c**), loricrin/involucrin (**d–f**), cytokeratin 1/cytokeratin 10 (**g–i**), occludin/E-cadherin (**k–m**). (**a**, **d**, **g**, **k**) Indirect immuno-fluorescence reveals the usual epidermal differentiation of KdES 3 weeks after transplantation. (**b**, **e**, **h**, **l**) Epidermal differentiation of SdES 3 weeks after transplantation. A delayed differentiation is obvious with respect to the expression pattern of K1/K10 (**h**). (**c**, **f**, **i**, **m**) Epidermal differentiation of SdES 7 weeks after transplantation. No difference from the control situation (compare with **a**, **d**, **g**, **k**) can be observed. White dotted line: dermo-epidermal border. Bar = 50 μm.

Periplakin was detected in all suprabasal layers, but was more prominent in the upper spinous and granular layers (Figure 5d and f, and Supplementary Figure S1b online). Once again, this pattern was not yet completely established in SdES after 3 weeks (Figure 5 e).

Integrin α6β4 has a central role in the binding of hemidesmosomes to laminin 332 (laminin 5), which is an important constituent of the basement membrane on which basal epidermal keratinocytes reside (Aumailley and Rousselle, 1999).

Integrin α6 (Figure 5d, e, and f) and laminin 332 (laminin 5, Figure 5g, h, and i), as well as laminin 511 (laminin 10, data not shown), were found to be expressed in the basement membranes of KdES and SdES. For both proteins the expression pattern did not differ from that of normal skin (for comparison, see Supplementary Figure S1b and g online). However, in SdES, homogeneous expression was delayed.

K5 is expressed in the basal layer and the first suprabasal layers in normal human homeostatic epidermis (Supplementary Figure S1g online). However, expression of K5 can be induced in migrating (activated) keratinocytes at wound edges (Patel *et al.*, 2006). Our analyses revealed that in KdES almost the normal staining pattern was found after 3 weeks (Figure 5g), whereas in SdES, K5 was strongly expressed suprabasally (Figure 5h). At 7 weeks after transplantation both types of ESs showed a K5 distribution that was similar to that of normal human epidermis (Figure 5i).

K15 is a useful indicator of epidermal homeostasis, as it is almost exclusively expressed in basal keratinocytes in a homeostatic epidermis (Pontiggia *et al.*, 2009). Indeed, it is absent during wound healing (Waseem *et al.*, 1999; Porter *et al.*, 2000). Consistently, K15 was neither expressed in keratinocytes and sweat gland cells grown on culture plastic, nor in KdES/SdES before transplantation. After transplantation K15 became upregulated in the stratum basale, which then

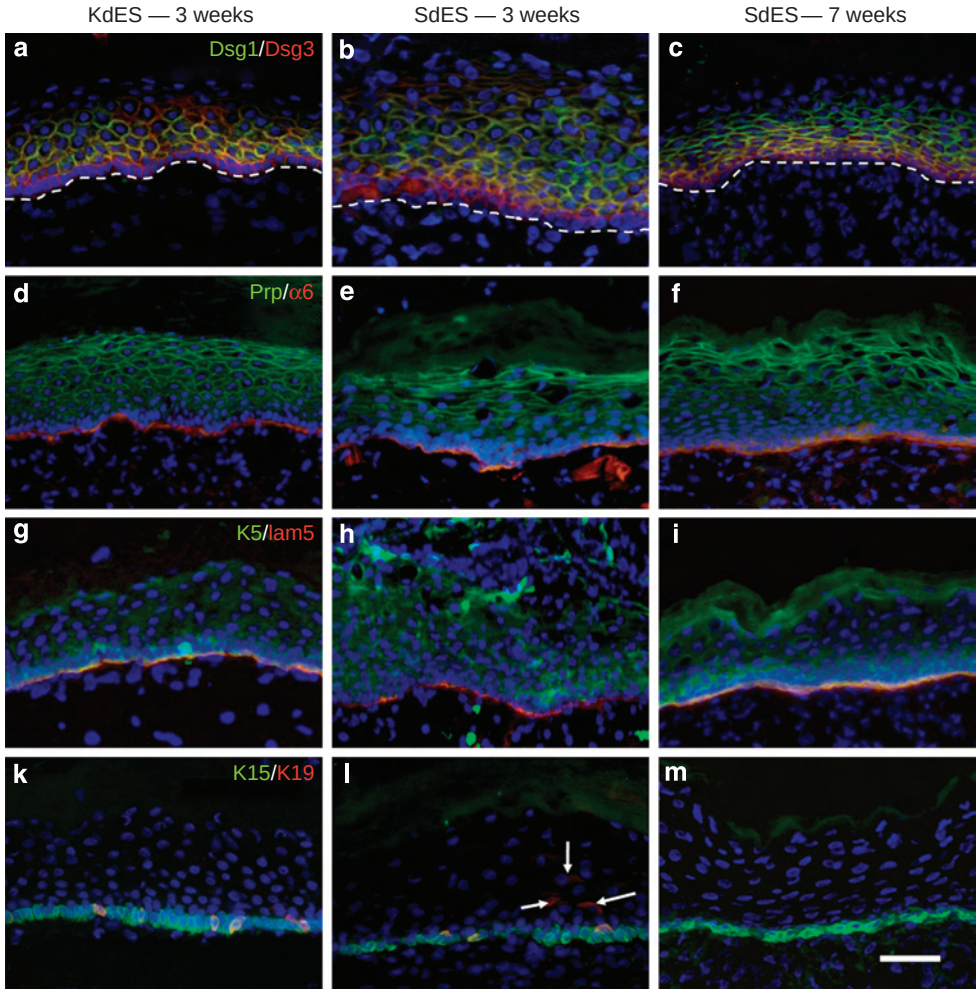

Figure 5. Epidermal stratification and homeostasis in keratinocyte-derived epidermal substitutes (KdESs) and sweat gland-derived epidermal substitutes (SdESs) after transplantation onto immuno-incompetent rats. Double immuno-fluorescence of cryosections using antibodies to: desmoglein (Dsg) 1/Dsg 3 (**a–c**), periplakin/integrin α6 (**d–f**), cytokeratin 5/laminin 5 (laminin 332) (**g–i**), and cytokeratin 15/cytokeratin 19 (**k–m**). (**a**, **d**, **g**, **k**) Indirect immuno-fluorescence shows the typical epidermal differentiation of KdES 3 weeks after transplantation. (**b**, **e**, **h**, **l**) Epidermal differentiation of SdES 3 weeks after transplantation. A delayed differentiation is visible with respect to the expression pattern of Dsg 1/Dsg 3 (**b**), integrin α6 (**e**), and K5 (**g**). Note that some K19-positive keratinocytes are still distributed suprabasally (white arrows in **l**). (**c**, **f**, **i**, **m**) Epidermal differentiation of SdES 7 weeks after transplantation. No difference from the control situation (compare with **a**, **d**, **g**, **k**) can be observed. K19-positive keratinocytes in the stratum basale have already disappeared, indicating advanced tissue homeostasis and differentiation. White dotted line: dermo-epidermal border. Bar = 50 μm.

exhibited the K15 expression pattern of normal human skin (Figure 5k, l, and m; compare with Supplementary Figure S1h online).

K19 is considered to be a marker of very ''young'', proliferative human skin (found in children from less than 2 years) (Pontiggia *et al.*, 2009). In normal interfollicular epidermis, K19-positive keratinocytes are clustered in the stratum basale as a subpopulation of K15-positive basal keratinocytes in young skin and disappear with age (Pontiggia *et al.*, 2009; Supplementary Figure S1h online). Figure 5k and l shows K19 expression in both KdESs and SdES 3 weeks after transplantation. Note that in Figure 5l some K19-positive keratinocytes are still distributed suprabasally, indicating that tissue homeostasis was not yet completely established. In 7-week-old transplants, K19 expression was already undetectable in both types of substitutes (Figure 5m). The expression patterns of all markers used in this study are summarized in Table 2.

Ultrastructural analysis of the ESs

Using transmission electron microscopy, we set out to study the two types of ESs on an ultrastructural level. Figure 6a shows an overview of an SdES, whereas the same structures in KdES are not shown. Quite frequently, blood vessels containing erythrocytes were evident (data not shown). The transplants were generally readily tolerated by the rats. Accordingly, macrophages and leukocytes in the dermal extracellular matrix were rare. Relatively large epidermal intercellular spaces, indicative of active fluid transport and occasional autophagosomes in the epithelial

Table 2. Expression of epidermal markers in KdES and SdES

Antigen	Antibody (clone)	Normal skin	KdES 3 weeks	SdES 3 weeks	SdES 7 weeks	
Occludin	Polyclonal	●●	●●	●	●●	
Loricrin	Polyclonal	●●	●	●	●	
Involucrin	SY5	●●	●	●	●	
Periplakin	Polyclonal	●●	●●	●●	●●	
K1	LHK1	●●	●●	●	●●	
K10	DE-K10	●●	●●	●●	●●	
K2e	Ks 2.342.7.4	●●	●●	(●)	(●)	
K16	LL025	—	●●	●●	●●	
K9	Polyclonal	—	—	—	—	Only in palmoplantar skin
Desmoglein 1	27B2	●●	●	●	●	
Desmoglein 3	5G11	●●	●	●	●	
E-Cadherin	NCH-38	●●	●●	●●	●●	
K5	Polyclonal	●●	●	●	●	
K15	LHK15, SPM190	●●	●●	●●	●●	
K19	RCK108	●●	●●	●●	—	
Integrin a6	4F10	●●	●●	●●	●●	
Laminin 5	P3H9-2	●●	●●	●●	●●	
Laminin 10	4C7	●●	●●	●●	●●	
Ki-67	B56	●●	●	●	●	
K8	B391	—	—	—	—	Only in secretory sweat gand cells
K4	6B10	—	—	—	—	Only in mucosal epithelium

Abbreviations: ●●, normal expression; ●, reduced expression; (●), expressed in single scattered cells; —, no expression; KdES, keratinocyte-derived epidermal substitute; SdES, sweat gland-derived epidermal substitute.

cells (asterisk in Figure 6b), denote a high metabolic turnover in the ESs.

Figure 6b illustrates a sweat gland-derived cell with clearly visible intermediate filament bundles (white arrow). This basal cell formed hemidesmosomes (large black arrows) connecting to the basement membrane (group of small black arrows). The complex organization of hemidesmosomes is illustrated in Figure 6c. In the spinous layer, sweat gland cells interconnected to one another via desmosomes (Figure 6d). All structures indicative of a normal cornified envelope can be detected (Figure 6e): In the upper spinous layer, cells flattened during keratinization and became considerably smaller. Keratohyalin granules (Figure 6e, arrows) were detected in the cells of the granular layer. They increased in number and size toward the stratum granulosum, whereas the nuclei disappeared. Finally, an electron-dense stratum corneum, consisting of flattened cells devoid of organelles, as well as detaching squames were evident. Figure 6f shows the magnification of a lamellar body in contact with the plasma membrane.

Taken together, transmission electron microscopy analyses of SdES revealed that human eccrine sweat gland cells can form a stratified ES, which, ultrastructurally, is indistinguishable from a KdES.

DISCUSSION

A central issue of engineering human skin substitutes is the number and quality of epidermal keratinocyte stem cells present in the keratinocyte preparation. A distinct keratinocyte stem cell pool, referred to as the epidermal proliferative unit (EPU), is commonly thought to be located in the epidermal stratum basale (Ghazizadeh and Taichman, 2005; Kaur, 2006; Strachan and Ghadially, 2008). An additional source of self-renewing keratinocytes is the hair follicle (Alonso and Fuchs, 2003; Cotsarelis, 2006; Blanpain and Fuchs, 2009). However, there is evidence that keratinocyte stem cells are only released from the hair follicle bulge if induced by epidermal wounding (Ito *et al.*, 2005; Levy *et al.*, 2005, 2007). The fact that there are regions of the human body that are devoid of hair follicles, such as palmo-plantar skin, indicate that hair follicles are not an exclusive source for keratinocyte stem cells.

An additional appendage of human skin is the eccrine sweat gland. Almost the entire human body is covered with sweat glands. One may therefore ask whether, in analogy to hair follicles, sweat glands can give rise to interfollicular keratinocytes in wound situations, or even serve as a permanent source of interfollicular keratinocyte stem cells (see also Figure 7).

Using a combined *in vitro*/*in vivo* bioassay, we demonstrated that ESs can be generated from sweat gland-derived epithelial cells. These substitutes can be further differentiated and maintained in homeostasis on experimental animals for at least 2 months. They exhibit almost all the properties of an epidermis derived from *a priori* epidermal keratinocytes (with

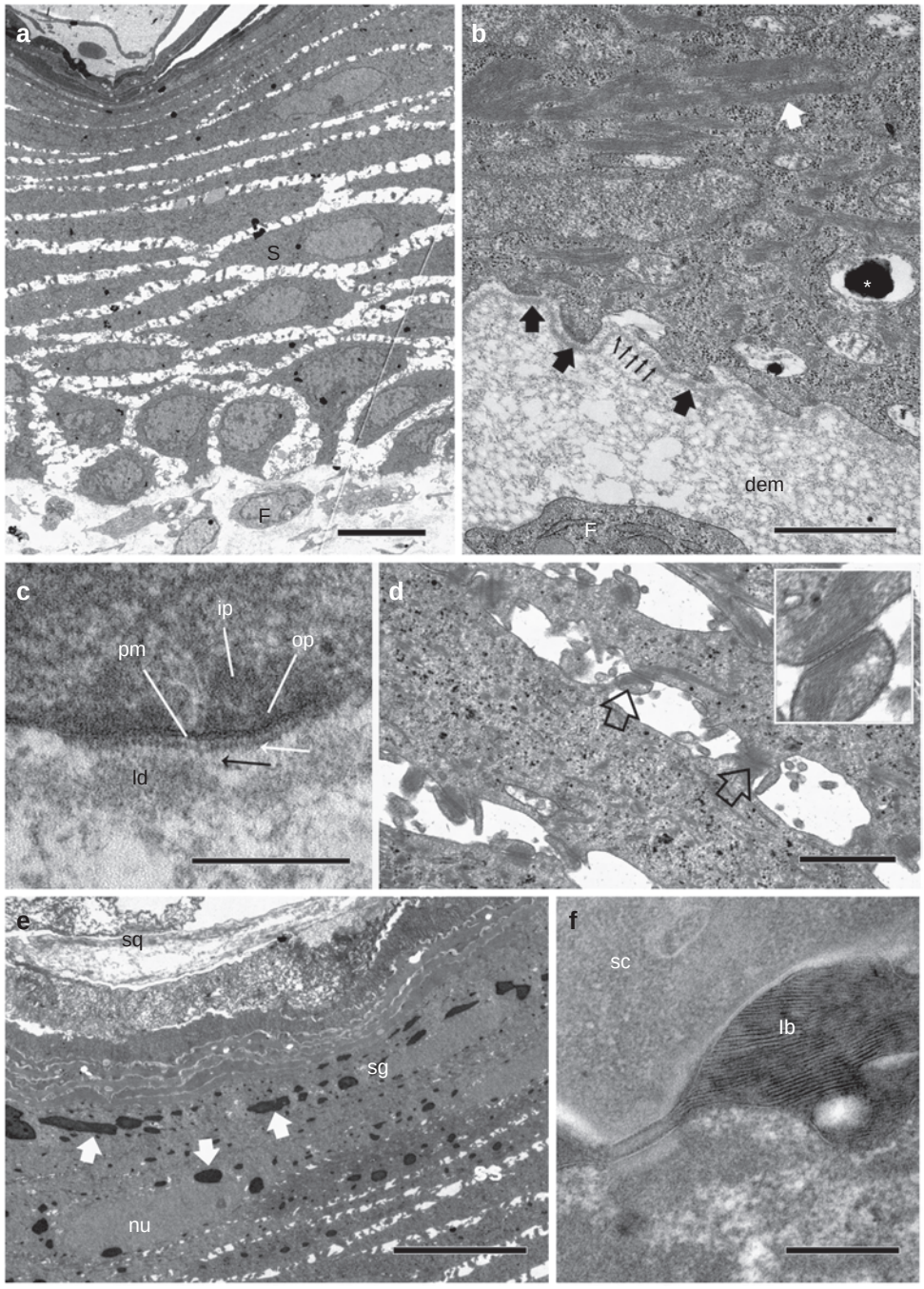

Figure 6. Transmission electron microscopy of a sweat gland-derived epidermal substitute (SdES) after transplantation reveals that sweat gland-derived cells develop into characteristic human epidermal structures. (**a**) The typical epidermal stratification and the characteristic adhesion of epidermal keratinocytes by numerous desmosomes are obvious. The lower pale cells are dermal fibroblasts (F). Dark cells are sweat gland-derived cells (S). (**b**) A mature dermo-epidermal junction has developed. A basal lamina (group of small arrows) and hemidesmosomes (black filled arrows) as well as the keratin filament network (white single arrow) and an autophagosome (asterisk) in an epithelial cell are indicated. (**c**) The complex structure of a hemidesmosome is shown. A complete basal lamina is deposited consisting of the sublamina dense plate (white arrow), the lamina lucida (black arrow), and the lamina densa (ld). (**d**) Mature desmosomal connections (black arrows) between adjacent epithelial cells of the spinous layer. The inset depicts a typical desmosome at a higher magnification. (**e**) Magnification of the upper epidermal layers showing the stratum spinosum (ss), the stratum granulosum (sg) containing the dark keratohyalin granula (white arrows), the stratum corneum (sc), and skin squames (sq). (**f**) A lamellar body (lb) between two corneocytes in the sc is depicted. (**a**, **e**) Bar = 10 μm; (**b**) bar = 5 μm; (**d**) bar = 1 μm; (**c**, **f**) bar = 0.2 μm. dem, dermal extracellular matrix; F, fibroblast; ip, inner plate; op, outer plate; pm, plasma membrane.

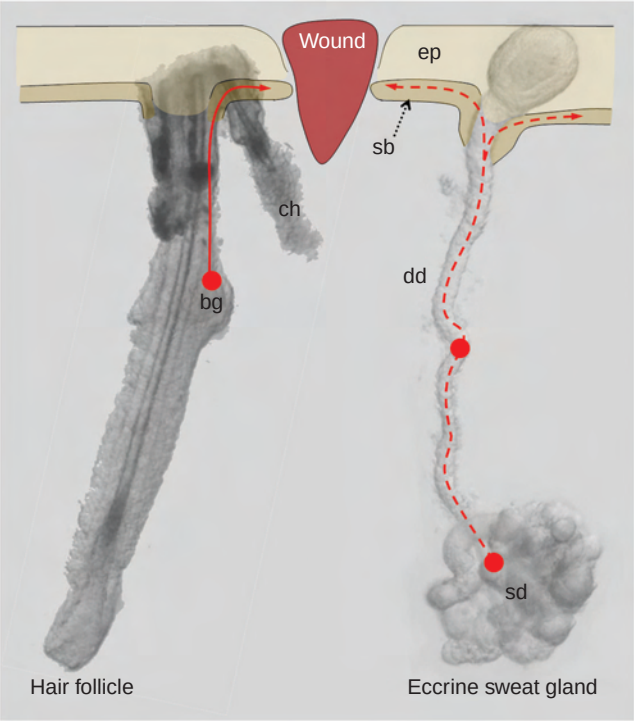

Figure 7. Hypothetical scheme considering both the human eccrine sweat gland and the human hair follicle as sources of epidermal keratinocyte (stem) cells. The human hair follicle bulge (bg) gives rise to epidermal keratinocytes that migrate toward the epidermis as it is locally injured upon wounding. As eccrine sweat gland cells have the general potential to generate an epidermis (ep), sweat gland cells derived from the secretory domain (sd) or ductal domain (dd) may also migrate toward the epidermal stratum basale (sb) to participate in closing an epidermal wound. It may even be considered that sweat gland cells are involved in the homeostatic maintenance of the human epidermis. ch, club hair.

the exception of K2e expression, which appears to be retarded). This is substantiated by the following findings: (1) a stratified epidermis consisting of 10–12 cell layers is formed by sweat gland cells; (2) a distinct, anuclear stratum corneum develops and is maintained after transplantation of SdES *in vivo*; (3) proteins centrally involved in the process of cornification, such as filaggrin, loricrin, involucrin, envoplakin, periplakin, and the transglutaminases I and III, are expressed in a pattern that matches that of normal human skin; (4) junctional complexes and hemidesmosomes are readily and regularly established; (5) K15 and K19-expressing keratinocytes become expressed in a distinct stratum basale 3 weeks after transplantation (K19-expressing cells disappear 7 weeks after transplantation.); (6) cell proliferation in the basal layer reaches homeostatic levels; (7) the sweat gland-derived epidermis is anchored within a well-developed basal lamina; (8) palmo-plantar or mucosal markers are not expressed in the sweat gland-derived epidermis; (9) similar to KdESs, engineered SdESs undergo maturation after their transplantation onto a living organism.

It was important to have the sweat gland preparations free of epidermal keratinocytes. This was achieved by using the micro-dissection technique, which was based on optical inspection of every single sweat gland, allowing the careful exclusion of the upper ''epidermal'' duct in all of the prepared sweat glands. The absence of epidermal tissue was confirmed by showing that K2e-positive keratinocytes, constituting 80–90% of the cells of the human epidermis, were absent in the sweat gland preparations. In addition, we provided indirect indication that no cells of the epidermal stratum basale were ''contaminating'' the sweat gland cell fraction. As there was no reliable antibody that allowed to discriminate between basal epidermal keratinocytes and sweat gland cells, this was done by demonstrating that epidermal melanocytes were absent in the sweat gland cell fraction. Taken together, these lines of evidence strongly suggest that a pure sweat gland cell population, consisting of secretory and ductal cells, has the potential to develop into a functional, stratified epidermis.

Although SdESs exhibited almost the complete set of epidermal markers and all the features of epidermal differentiation (see also Table 2), we realized a developmental delay in the expression of these markers and the establishment of epidermal homeostasis when compared with KdESs. This is most likely due to sweat gland cells undergoing a time-consuming transition from ductal or secretory glandular cells to interfollicular epidermal keratinocytes. Thus, the delayed expression of markers such as K1, K10, K5, K19, and occludin in SdES is not really surprising. In addition, myoepithelial cells that were co-isolated with secretory sweat gland cells may have a stabilizing effect on the sweat gland cell phenotype in the organism. In our experimental setting, myoepithelial cells relatively rapidly disappeared in the cell cultures. Thus, the loss of myoepithelial cells, which are closely associated with secretory sweat gland cells, in combination with the massive cell dissociation (representing an extreme wound situation) and the subsequent proliferation on cell culture plastic, may be the prerequisite for the transition of sweat gland cells into epidermal keratinocytes.

Sweat gland cells are derived from ectodermal cells that differentiated into ductal and secretory glandular cells during embryogenesis (Fu *et al.*, 2005). The cell transition described in this paper may represent the reversion of this ontogenetic process. There are two conceivable mechanisms:

1. A multipotent, sweat gland-derived stem cell population proliferates and the resulting new cells differentiate in response to their new epidermal environment.
2. Sweat gland cells might de-differentiate into a more naive state, after which they differentiate into a new phenotype, i.e., epidermal keratinocytes. Based on the fact that the new sweat gland-derived epidermis reached a state of homeostasis, and was permanently maintained *in vivo*, it has to be postulated that self-renewing cells assured the integrity of the new epidermis. We consider it therefore likely that both re-programmed sweat gland-derived cells and sweat gland-derived stem cells in concert formed and maintained the new epidermis.

Interestingly, under no experimental circumstances did sweat gland-derived epithelial cells develop into sweat gland-like structures. This suggests that the observed cell transition follows a default pathway of differentiation, which invariably

causes sweat gland cells to convert into epidermal keratinocytes. This pathway may well be of regulatory relevance. It is consistent with the concept that, in analogy to keratinocytes derived from human hair follicle bulge, a self-renewing subpopulation of sweat gland cells has the potential to reconstitute human epidermis upon wounding, or even to permanently participate in regenerating it (for an illustration, see Figure 7). As glabrous skin (such as the palms and soles) is rich in sweat glands (Frinkel and Woodley, 2001), the presence of a self-renewing, potentially epidermal cell population in sweat glands was repeatedly postulated (Brouard and Barrandon, 2003; Barrandon, 2007). The data presented in this paper provide evidence that significantly supports this assumption. To our knowledge this is previously unreported.

It remains to be investigated which subtype of sweat gland cells, secretory and/or ductal cells, is involved in epidermal regeneration and maintenance. Some recent evidence suggests the presence of a label-retaining, multi-potent stem cell population in the secretory domain of the human eccrine sweat gland (Nakamura and Tokura, 2009). Apart from that, only few data can be found in the literature that support the ability of the human sweat gland cells to form an epithelium in culture and *in vivo*. Miller *et al.* (1998) reported that in pigs the ''sweat apparatus'' can re-epithelialize the skin ''*de novo*'' after wounding. These authors concluded that porcine sweat gland cells formed an epithelium that had the morphological and structural features of palmo-plantar or buccal epithelia, rather than the features of trunk epidermis. However, in contrast to human skin, which contains eccrine sweat glands, porcine skin harbors apocrine glands, the function of which is different from that of human sweat glands (Montagna and Yun, 1964; Ferry *et al.*, 1995).

In conclusion, this is the first study generating comprehensive evidence that a near-normal epidermis can be cultured from sweat gland cells *in vitro*. Furthermore, the SdES can be successfully transplanted onto immuno-incompetent rats on which they adhere, further differentiate, and survive as a mature graft, which is strikingly similar to the KdES. This study enlarges the understanding of skin biology in that it corroborates the thesis that sweat gland cells can switch their phenotype and become keratinocytes. From a tissue engineering point of view, sweat gland-derived epithelial cells obviously represent an additional source of keratinocytes to grow a near-normal autologous epidermis. This is particularly relevant for patients requiring large and urgent covering of skin defects, such as severe burn injuries (third degree; >50% BSA). In these cases, self-renewing keratinocytes are urgently required for the *in vitro* production of as many life-saving skin grafts as possible.

MATERIALS AND METHODS

Preparation of skin cells

This study was conducted according to the Declaration of Helsinki Principles. Human skin samples from scalp, abdomen, retroauricular skin, or foreskins were obtained from patients ranging in age between 1 and 18 years, after obtaining permission from the ethics commission of the Canton Zurich, and after informed consent was given by parents or patients. The skin samples were used for the isolation of sweat gland cells, keratinocytes, and fibroblasts, or for histological analysis.

Establishment of primary cell cultures

Keratinocytes and fibroblasts were isolated as described (Pontiggia *et al.*, 2009). For sweat gland cells, we used the following procedure.

On the day before isolation, mitomycin-treated or irradiated Swiss albino 3T3 mouse fibroblasts (ATCC CCL-92) were seeded at a density of 10,000 per cm^2 in DMEM, 10% fetal calf serum, and 5 $\mu g\,ml^{-1}$ gentamycin. Just before plating the fragments of sweat glands, the medium was changed to Rheinwald and Green (Jones *et al.*, 1988; Schon *et al.*, 1999), i.e., three parts of DMEM and one part of Ham's F12, 10% fetal calf serum, 5 $\mu g\,ml^{-1}$ gentamycin, 1.4 mM $CaCl_2$ (all from Sigma, Buchs, Switzerland), 0.4 $\mu g\,ml^{-1}$ hydrocortisone, 5 $\mu g\,ml^{-1}$ insulin, 2 nM triiodothyronine, 180 μM adenine, 10 $ng\,ml^{-1}$ EGF (all from Invitrogen, Basel, Switzerland), and 0.1 nM choleratoxin (Calbiochem/VWR International AG, Dietikon, Switzerland). Skin samples were cut into small pieces (~6 mm^2), digested in DMEM containing 12 $U\,ml^{-1}$ dispase (Invitrogen), 2 $mg\,ml^{-1}$ collagenase blend F (Sigma), and 38 $U\,ml^{-1}$ collagenase II (Worthington, Bioconcept, Allschwil, Switzerland) for 16 hours at 4 °C and for 4 hours at 37 °C until the mesenchyme was almost completely digested. The tissue pieces were centrifuged and the fat droplets were removed with the supernatant. The pellet was resuspended in 20 ml of DMEM containing threefold concentrated antibiotics (gentamycin 15 $\mu g\,ml^{-1}$, penicillin 3,000 $U\,ml^{-1}$, streptomycin 3 $mg\,ml^{-1}$, and fungizone 750 $ng\,ml^{-1}$) and 1% fetal calf serum, and transferred to a new culture dish. The dermal tissue was removed from the epidermis and fragments of sweat glands were collected using a stereomicroscope and micropipettes. The fragments were washed with culture medium and finally transferred to the culture dish. At days 4–5 the first outgrowing cells were visible. After 2 weeks the feeder cells were removed by digestion with 0.1% trypsin and 1 mM EDTA (Invitrogen) for 2 minutes at 37 °C. The remaining sweat gland cells were washed twice in phosphate-buffered saline (PBS) and detached from the dish with 0.5% trypsin and 5 mM EDTA for 5 minutes at 37 °C. Trypsin activity was stopped by the addition of 3.75 $mg\,ml^{-1}$ soy bean trypsin inhibitor (Invitrogen). An almost single-cell suspension was reached by repeated pipetting through a fine pipette tip.

Keratinocytes and sweat gland cells were further expanded during passage 1. Keratinocytes were grown in a serum-free medium (Invitrogen, containing 0.2 $ng\,ml^{-1}$ EGF and 25 $\mu g\,ml$ bovine pituitary extract). Sweat gland cells were maintained in Rheinwald and Green medium.

Organotypic cultures

Organotypic cultures were prepared using a previously established transwell system consisting of six-well cell culture inserts with membranes of 3 μm pore size (BD Falcon, Basel, Switzerland; Pontiggia *et al.*, 2009). The membranes were covered with collagen type I hydrogels containing human dermal fibroblasts (passage 1).

The collagen matrix was prepared according to the protocol of Costea *et al.* (2003). Briefly, 0.7 ml of rat tail collagen type I (3.2–3.4 $mg\,ml^{-1}$, BD Biosciences, Allschwil, Switzerland) was added to 0.2 ml of chilled neutralization buffer containing 0.15 M

NaOH and 1×10^5 fibroblasts. After polymerization (10 minutes at room temperature and 20 minutes at 37 °C), these dermal equivalents were grown in DMEM/10% fetal calf serum for 5 days. Subsequently, keratinocytes and sweat gland cells were seeded onto each dermal equivalent at a density of 125×10^3 cells per cm^2 within siliconized polypropylene rings of 5 mm diameter to avoid dispersion. After 7 hours the rings were removed, 1 ml Rheinwald and Green medium was added to the upper chamber and 2 ml to the lower chamber. Triplicate wells were set up for each dermo-epidermal substitute. The constructs were cultured in Rheinwald and Green medium. After 4 days the dermo-epidermal substitutes were raised to the air–liquid interface and cultured for three additional weeks. The medium was changed every second day. Cultures were finally processed for transplantation or for cryo and paraffin sections.

Transplantation of cultured dermo-epidermal composites

Dermo-epidermal grafts were transplanted onto full-thickness skin defects created surgically and encased by polypropylene rings, 27 mm in diameter, as previously described (Pontiggia *et al.*, 2009). The rings were sutured on the back of 10-week-old, female athymic Nu/Nu rats. The transplants were covered with a silicon foil. After 14 days the grafts were excised and processed for cryo and paraffin sections. Anesthesia for all procedures was performed using isoflurane (Abbott AG, Baar, Switzerland).

Histology and Immuno-fluorescence

The ESs were embedded in an optimal cutting temperature compound (Sakura Finetek/Digitana AG, Horgen, Switzerland) and frozen at −20 °C. Melanin was visualized using the Masson–Fontana technique with nuclear fast red as counterstain (Stevens and Chalk, 1996).

Cryosections were fixed and permeabilized in acetone for 5 minutes at −20 °C, air dried, washed three times in PBS, and blocked in PBS containing 2% BSA (Sigma) for 30 minutes. Incubation with the diluted antibodies was performed in blocking buffer for 1 hour at room temperature. Slides were washed three times for 5 minutes in PBS and blocked for an additional 15 minutes before the second antibody was added. If necessary, the same procedure was repeated for the third antibody incubation. Finally, the slides were incubated for 5 minutes in PBS containing 1 μg ml^{-1} Hoechst 33342 (Sigma), washed twice for 5 minutes in PBS, and mounted with Dako mounting solution (Dako, Baar, Switzerland) containing 25 mg ml^{-1} of DABCO anti-quenching agent (Sigma).

To stain freshly isolated sweat gland cells, the glands were dissociated for 3 minutes by means of concentrated (3 ×) trypsin/EDTA (Invitrogen). After inactivation of trypsin, the cells were fixed in 2% paraformaldehyde for 10 minutes, permeabilized with 0.5% saponin for 5 minutes, and incubated with the primary antibodies for 15 minutes on ice. The cells were passed through a 0.2-ml serum layer by centrifugation and resuspended in PBS containing 2% BSA and the fluorescent-dye-conjugated secondary antibody for 15 minutes on ice. After the second serum-washing step, the cells were centrifuged onto glass slides and the cytospins were analyzed by fluorescence microscopy.

Antibodies

For immuno-fluorescence the following antibodies were used—from Dako: K10 (clone DE-K10, 1:100), E-cadherin (clone NCH-38, 1:30), laminin 10 (clone 4C7, 1:25), K19 (clone RCK108, 1:100), melanosome (clone HMB45, 1:50); from Progen (Heidelberg, Germany): K2e (clone Ks 2.342.7.4, 1:100), K9 (polyclonal, 1:200), K5 (polyclonal, 1:100); from Chemicon (Millipore AG, Zug, Switzerland): K16 (clone LL025, 1:100), K1 (clone LHK1, 1:200), integrin α6 (clone 4F10, 1:100), K15 (clone LHK15, 1:100); from Zymed (Invitrogen): occludin (polyclonal, 1:50), Dsg 1 (clone 27B2, 1:50), Dsg 3 (clone 5G11, 1:200); from LabVision (PH Stehelin&CIE AG, Basel, Switzerland): involucrin (clone SY5, 1:100), filaggrin (FLG01, 1:100); from Genetex (Biozol, Eching, Germany): K8 (clone B391, 1:200); from Sigma: K4 (clone 6B10, 1:600); from BD Pharmingen (Basel, Switzerland): Ki-67 (clone B56, 1:200); from Abcam (Cambridge, UK): loricrin (polyclonal, 1:500); from Santa Cruz (Labforce AG, Nunningen, Switzerland): laminin 5 (clone P3H9-2, 1:100). The periplakin and envoplakin antibodies were kind gifts from Dr Fiona Watt (Cambridge Research Institute, Cambridge, UK). As a secondary antibody we used FITC-conjugated polyclonal goat $F(ab')_2$ fragments directed to mouse immunoglobulins (Dako). For double immuno-fluorescence, some of the primary antibodies were pre-labeled with Alexa 555-conjugated polyclonal goat F(ab′)2 fragments, according to the manufacturer's instructions (Zenon Mouse IgG Labeling Kit, Molecular Probes, Invitrogen).

Fluorescence microscopy

Fluorescence microscopy was performed using a Nikon Eclipse TE2000-U inverted microscope (Nikon AG, Egg, Switzerland) equipped with Hoechst, FITC, and tetramethylrhodamine isothiocyanate filter sets. For confocal imaging, we used a Leica SP1 confocal laser scanning microscope (Leica, Heerbrugg, Switzerland) equipped with argon UV laser with 351 nm/364 nm excitation, argon/krypton laser with 474 nm/488 nm/568 nm/647 nm excitation, and fluorescence filters for 4′,6-diamino-phenylindole, FITC, and tetramethylrhodamine isothiocyanate. The line average was set to 4. Images were processed with Imaris 5.0.1 (Bitplane AG, Zurich, Switzerland).

Electron microscopy

For transmission electron microscopy analysis, tissue blocks (approximately 1 mm^3) were prefixed in 0.1 M cacodylate buffer (Merck, Hohenbrunn, Germany), pH 7.3 containing 2.5% glutaraldehyde for 2 hours, washed in cacodylate buffer, postfixed with an aqueous solution of 1% OsO4 and 1.5% K4Fe(CN)6 for 1 hour, dehydrated, and finally embedded in EPON 812 (Catalys AG, Wallisellen, Switzerland). Ultrathin sections (approximately 50–70 nm) collected on copper grids were contrasted with 4% uranyl acetate and 3% lead citrate, and examined with a CM 100 transmission electron microscope (Philips, Eindhoven, The Netherlands). All reagents were from Sigma unless mentioned otherwise.

In situ Hybridization with Alu probes

The ESs were fixed in 4% neutral formalin and embedded in paraffin according to standard protocols. We used the method published by Just *et al.* (2003). Briefly, the genomic DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Hombrechtikon, Switzerland) from the whole blood of a volunteer who donated 2 ml blood after giving written informed consent. In all, 50 ng of genomic DNA and 0.5 μM of the primers (Alu sense: 5′-ACGCCTGTAATCCCAGCACTT-3′; Alu antisense: 5′-TCGCCCAGGCTGGAGTGCA-3′ produced by Microsynth, Balgach, Switzerland) were applied in a PCR with Hot

Star Taq DNA polymerase (Qiagen) under the following conditions: 95 °C for 15 minutes, 25 × (94 °C for 30 seconds, 58 °C for 45 seconds, and 72 °C for 45 seconds), and 72 °C for 10 minutes. The 245-bp product was purified by agarose gel electrophoresis and using the QIAquick Gel Extraction Kit (Qiagen). An amount of 200 pg was used as template for the digoxygenin (DIG)-labeling PCR (PCR DIG probe synthesis kit, Roche, Rotkreuz, Switzerland) under the same conditions, but with increased $MgCl_2$ concentration (5 mM). The DIG-labeled probe was purified by ethanol precipitation.

Paraffin sections were mounted on superfrost slides, deparaffinized (xylol, 3 × 10 minutes), hydrated (ethanol 100–96–80–70–50%, 2 minutes each), and washed in PBS (3 × 5 minutes). Cryosections (16 μm thick) were mounted on superfrost slides, dried for 30 minutes at 37 °C, washed in PBS (3 × 5 minutes), fixed with 4% paraformaldehyde for 20 minutes and washed again. Both section types were further permeabilized (0.3% Triton X-100 in PBS, 10 minutes), digested with proteinase K (2 μg ml^{-1} in 100 mM Tris-HCl, pH 8, 50 mM EDTA, 15 minutes at 37 °C; for cryosections 5 minutes only), acetylated (0.1 M triethanolamine, 0.25% acetic anhydride, 2 × 5 minutes), and washed first in PBS (2 × 5 minutes) and finally in 2 × SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0, 5 minutes). The hybridization was performed in a moist chamber with 50% formamide. The slides were incubated in the hybridization buffer (50% formamide in 5 × SSC, 0.15% *N*-lauroylsarcosine, 0.02% SDS, 2% blocking reagent) without probe for 30 minutes at 85 °C, and with 100 ng/ml DIG-labeled DNA probe for 30 minutes at 85 °C. After chilling on ice for 10 minutes, the slides were incubated overnight at 42 °C and finally washed in 2 × SSC (briefly) and 0.1 × SSC (3 × 15 minutes at 42 °C). For staining, the sections were blocked (30 minutes in 0.15 M NaCl, 0.1 M maleic acid, pH 7.5, 1% blocking reagent), incubated with an alkaline phosphatase-conjugated anti-DIG antibody (1:2,000 in blocking buffer and 0.1% Triton X-100, 60 minutes), washed (4 × 15 minutes in 0.15 M NaCl, 0.1 M maleic acid, pH 7.5), equilibrated in Tris-buffered saline (0.1 M Tris, pH 9.5, 0.1 M NaCl, 5 minutes), incubated with the substrate solution (200 μl of Nitroblue tetrazolium salt + bromo-chloro-indolyl phosphate in 10 ml Tris-buffered saline, 120 minutes) and the stop solution (10 mM Tris, pH 8.0, 1 mM EDTA, 2 minutes), and washed in PBS (3 × 15 minutes, second wash containing 1 μg ml^{-1} Hoechst 33342). Finally, the slides were mounted in Eukitt quick-hardening mounting medium. All reagents were from Fluka (Buchs, Switzerland); the proteinase K and the DIG nucleic acid detection kit, including the blocking reagent and substrate solution, were from Roche.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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